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Utilization of the Rabbit Alveolar Macrophage and Chinese Hamster Ovary Cell for Evaluation of the Toxicity of Particulate Materials. II. Particles from Coal-Related Processes

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16. ABSTRACT

Rabbit alveolar macrophage (RAM) and Chinese hamster ovary (CHO) cells were used in vitro tests to evaluate the toxicity of particulate effluents from coal gasification, fluidized-bed combustion, and conventional coal combustion. Evaluation of the cytotoxicity of nine samples from coal energy-related processes showed that the sensitivity of the RAM assay was improved substantially when the test was conducted in serum-free media. A linear relationship was observed between percentage cell viability and ATP level in the particle-treated cultures. In the RAM assay without serum the slope of the line was 1.0, which provided strong evidence that the mechanism of toxicity was similar in reducing cell viability and ATP. The cytotoxicity of the coal-related particles in the CHO clonal assay showed that the system was approximately equivalent to the RAM assay without serum. Conventional coal combustion fly ash ($< 3\ \mu m$) was the most toxic of the coal-related particles in the RAM and CHO system. Fluidized-bed combustion fly ash from a cyclone catch and fine ($< 3\mu m$) particles from the flue gas were also toxic in both cellular assays. Particles from the coal gasification process were the least cytotoxic in these studies.

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The Utilization of the Rabbit Alveolar Macrophage and Chinese Hamster Ovary Cell for Evaluation of the Toxicity of Particulate Materials

II. Particles from Coal-Related Processes

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Rabbit alveolar macrophage (RAM) and Chinese hamster ovary (CHO) cells were used in *in vitro* tests to evaluate the toxicity of particulate effluents from coal gasification, fluidized-bed combustion, and conventional coal combustion. Evaluation of the cytotoxicity of nine samples from coal energy-related processes showed that the sensitivity of the RAM assay was improved substantially when the test was conducted in serum-free media. A linear relationship was observed between percentage cell viability and ATP level in the particle-treated cultures. In the RAM assay without serum the slope of the line was 1.0, which provided strong evidence that the mechanism of toxicity was similar in reducing cell viability and ATP. The cytotoxicity of the coal-related particles in the CHO clonal assay showed that the system was approximately equivalent to the RAM assay without serum. Conventional coal combustion fly ash ($<3\ \mu m$) was the most toxic of the coal-related particles in the RAM and CHO system. Fluidized-bed combustion fly ash from a cyclone catch and fine ($<3\ \mu m$) particles from the flue gas were also toxic in both cellular assays. Particles from the coal gasification process were the least cytotoxic in these studies.

INTRODUCTION

The emission of particulate matter into the atmosphere from stationary fuel combustion sources utilized in industrial or energy-related processes poses a serious environmental problem. The amount and properties of combustion particles (fly ash) generated by stationary fuel combustion, and aspects of the technology used to control the emission of these pollutants have been reviewed (Abbott and Drehmel, 1979). Natusch and co-workers (1978) have shown that trace elements and chemicals associated with particulate matter from coal combustion may constitute a potential health hazard, and, in an earlier study (1974), reported that many known or potential toxic agents are preferentially concentrated on the surface of respirable coal fly ash. According to Davison *et al.* (1974), greater quantities of trace elements are also associated with the smallest particles of fly ash. The particles are often too small to be trapped effectively by conventional particulate control devices and are especially hazardous due to their respirable size.

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Several cytotoxicity assays have recently been employed to evaluate particulate emissions from industrial and energy-related sources in pilot studies for the EPA Environmental Assessment Program (Duke *et al.*, 1977). The use of rabbit alveolar macrophage (RAM) and Chinese hamster ovary cell (CHO) assay systems for evaluation of the toxicity of some particulate materials has been reported previously (Mahar, 1976; Garrett *et al.*, 1979; Garrett *et al.*, 1981). This paper describes the utilization of the RAM and CHO cell systems for evaluation of the cytotoxicity of particles from coal-related processes.

MATERIALS AND METHODS

Particles from coal gasification, fluidized-bed combustion, and conventional coal combustion processes were obtained through the Industrial Environmental Research Laboratory of the Environmental Protection Agency at Research Triangle Park, N.C. Samples from coal gasification (CG) processes included gasifier ash, coal feed, and dust collected from a cyclone (Radian Corporation, 1978). The fluidized-bed combustion (FBC) samples included the coal feed, dolomite sorbent feed, the sorbent bed discard or spentbed, fly ash from the second-stage cyclone catch, and a fine (<3 μm SASS cyclone catch) particulate from atmospheric stack or flue gas (Murthy *et al.*, 1979). The conventional coal combustion (CCC) particulate was a fly ash sample (<3 μm) collected on an electrostatic precipitator. An independent size analysis of this group of particles has been conducted (Garrett, 1980).

The samples were evaluated using the rabbit alveolar macrophage (RAM) and the Chinese hamster ovary (CHO) cell systems. Detailed aspects of the cellular and biochemical analyses, sample handling, and culture and treatment of cells were performed as reported previously (Garrett *et al.*, 1981). In brief, in the RAM assay, cells were added to individual wells of cluster dishes containing suspensions of the particulate samples. Experimentals and controls were randomly distributed among the total number of wells inoculated. Cells were harvested after a 20-hr exposure period. Cell viability was determined by exclusion of trypan blue, and the number of cells was determined by optical enumeration with a hemocytometer. Adenosine triphosphate (ATP) was assayed by luminescence using the luciferin–luciferase reaction (Waters *et al.*, 1975b). Total cell protein was determined using the Lowry method (1951) with bovine serum albumin as a standard. In the CHO cell assay 200–300 cells were plated from a dilute suspension into tissue culture flasks and allowed to attach for 18–24 hr. Replicate flasks were then exposed to samples and after 6 days colonies were fixed, stained, and enumerated (Wininger *et al.*, 1978).

Particulate samples were evaluated initially at a concentration of 1000 μg/ml. Dose–response curves were obtained for samples found to depress cell viability or ATP below 50%. All determinations were performed in triplicate for each experiment, and statistical analysis was performed on the cumulative data pool (generally six replicates). The data from dose–response experiments were used to determine an estimate of the sample concentrations needed to reduce cell viability, cell ATP or other parameters by 50% (EC_{50}). EC_{50} values were obtained from the dose–response relation by inverse prediction from the simple regression line.

All dose–response data were mathematically transformed and then fitted to a straight line by the method of least squares. Concentrations were expressed as the natural logarithm of the dose in micrograms per milliliter. Cell viability was normally transformed to the arc sine of the square root of the fraction of viable cells. ATP data expressed as a percentage of control were fitted to a straight line with the logarithm of the dose. Confidence limits on the estimated EC_{50} values were obtained using standard statistical procedures (Hald, 1952). The details of the data analysis were reported previously (Garrett and Stack, 1980).

RESULTS

The response of RAM cells to particles from coal-related processes after a 20-hr exposure at 1000 μg/ml of sample is shown in Fig. 1. It may be seen that the responses obtained from particles from the coal gasification (CG) process were not significantly different from the control ($P > 0.12$), but statistically significant differences were observed for the conventional coal combustion (CCC) fly ash ($P < 0.001$) and the fluidized-bed combustion (FBC) samples ($P < 0.001$) with the exception of particles from the dolomite sorbent bed. Cellular ATP was the most sensitive index of toxicity and was especially reduced for CCC fly ash and the FBC stack gas/fine particulate. Although the RAM assay provided a measure of discrimination of the toxicity of the particles, the sensitivity of the assay was not sufficient to adequately distinguish between individual particulates. Previous studies, with well-defined particulate substances and model fly ashes, showed that reduction of the serum content of the culture media enhanced the sensitivity of the

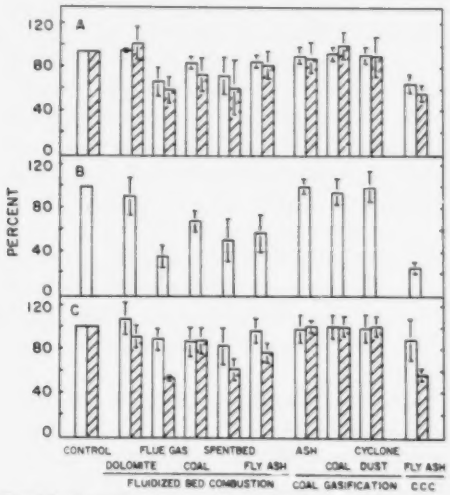

FIG. 1. Effects of fluidized-bed combustion, coal gasification, and conventional coal combustion particulates on RAM cells. Cells were exposed for 20 hr to 1000 μg/ml of particulate. The culture medium contained 10% fetal calf serum. Data are mean values ± standard deviation ($n = 6$). A: Cell viability, □, and viability index, ▨; B: ATP (fg/cell, percentage of control); and C: cells/ml, percentage of control, □, and cell protein, percentage of control, ▨.

RAM assay primarily by amplifying the effects on cell viability and ATP (Garrett *et al.*, 1981). In order to investigate the possibility that the RAM assay could be modified to better discriminate between environmental particles, the influence of serum concentration on the toxicity of CCC fly ash was studied (Fig. 2). In a manner similar to that previously reported for some model fly ashes, reduction of serum influenced the response of the macrophage. Cellular ATP, viability, viability index, and total cell protein decreased sharply as the serum content of the culture media was reduced. Cell number was also lowered when serum was completely removed indicating cell lysis.

Assay of the toxicity of the particles from coal related processes using the RAM system without fetal calf serum is shown in Fig. 3. The system without serum gave a substantial improvement in discrimination between individual samples. Cell viability and ATP were sharply reduced for the CCC sample and FBC fly ash and fine particulate samples. Cell numbers were minimally affected. Inspection of these data revealed that a well-defined relationship existed between cell viability and ATP. This relationship, shown in Fig. 4, was found to be linear with a correlation coefficient of 0.96; the slope of the line was 1.0, indicating that the particles exerted equivalent effects on cellular viability and ATP.

A linear relationship between cell viability and ATP was also found over most of the range of values if specific samples were evaluated at several concentrations (Fig. 5). These data show that a linear relationship was preserved when the assay was conducted using 10% serum; however, the slope and y intercept of the straight lines were altered. In the standard RAM assay cell ATP was a more sensitive index of toxicity than was cell viability; however, ATP and cell viability were

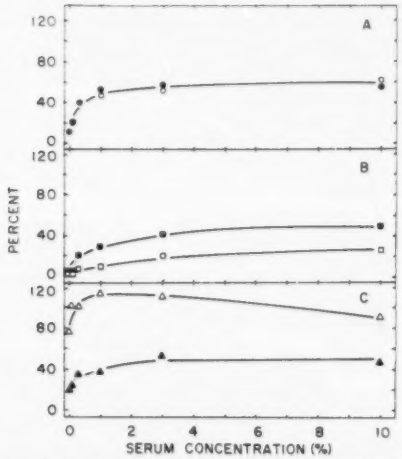

FIG. 2. Influence of fetal calf serum on the toxicity of conventional coal combustion fly ash. RAM cells were exposed to 1000 μg/ml of particles (0–3 μm). Each point represents the mean value for three replicate cultures. A: Cell viability, ○, and viability index, ●; B: ATP (fg/cell, percentage of control), □, and ATP (fg/protein, percentage of control), ■; and C: cells/ml, percentage of control, △, and cell protein, percentage of control, ▲.

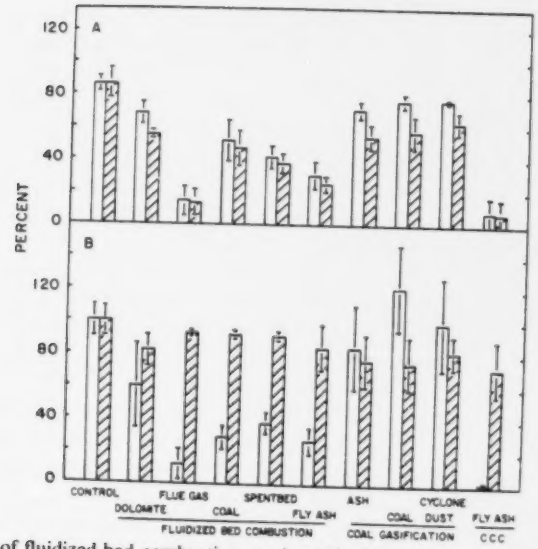

FIG. 3. Effects of fluidized-bed combustion, coal gasification, and conventional coal combustion particulates in the RAM bioassay without fetal calf serum. Cells were exposed for 20 hr to 1000 μg/ml of the particles. Data are mean values ± standard deviation ($n = 6$). A: Cell viability, □, and viability index, ▨; B: ATP (fg/cell, percentage of control), □, and cells/ml, percentage of control, ▨.

almost equivalent in sensitivity provided serum was withheld from the assay system.

An evaluation of the coal-related particles using the CHO clonal assay revealed that the response of the CHO system was essentially equivalent to the RAM assay

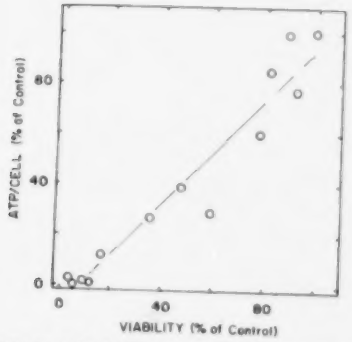

FIG. 4. The relationship between cell viability and ATP for coal-related particulates evaluated in the RAM assay. Cells were exposed for 20 hr to 1000 μg/ml of the particles using the assay without fetal calf serum. Data are presented for all the particles from the fluidized-bed combustion, coal gasification, and conventional coal combustion processes with the exception of coal particles from the gasification process. Data are also presented for model particulate materials including: fly ash (2–5 μm), lead oxide-coated fly ash (<2 μm), titanium dioxide particles (0.45 μm), and silicic acid.

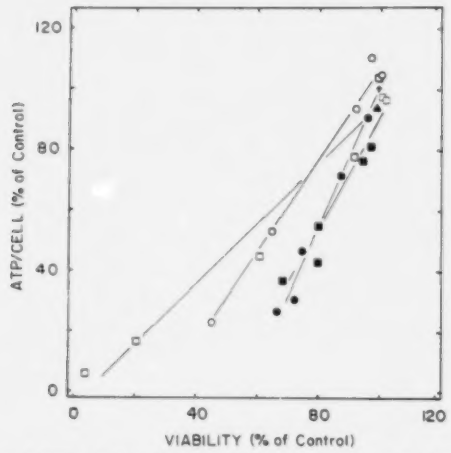

FIG. 5. Cell viability and ATP relationship for particles tested in the RAM assay. Control, †; stack gas particles with, ●, and without, ○, 10% fetal calf serum; and conventional coal combustion fly ash with, ■, and without, □, 10% fetal calf serum. Particles were tested at concentrations of 125–1000 μg/ml in experiments with 10% fetal calf serum, and at 10–500 or 1000 μg/ml without serum.

without serum (Fig. 6). In particular the data from the CHO and the RAM assay revealed the same three samples to be most toxic (FBC stack gas/fine particulate and fly ash, and the CCC fly ash). However, the CHO system was not as sensitive to the FBC coal and spentbed samples as was the modified RAM assay.

Data in Fig. 7 show that the dose–response relationship was also essentially equivalent in the RAM and CHO assay of the FBC stack gas/fine particulate. In addition, these data show that the leachate from the washed particulate was slightly toxic in the clonal assay.

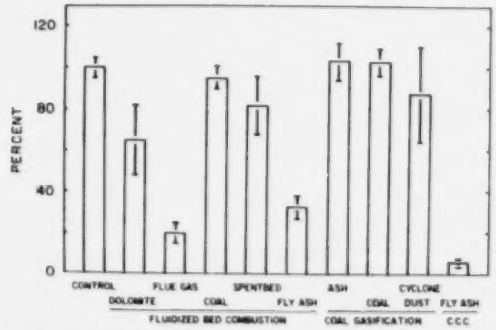

FIG. 6. The effect of fluidized-bed combustion, coal gasification, and conventional coal combustion particles in the CHO clonal assay. Data are expressed as a percentage of the control colony growth after a 6-day exposure. Cultures were exposed to 1000 μg/ml of the particles. Mean values of two experiments ($n = 6$).

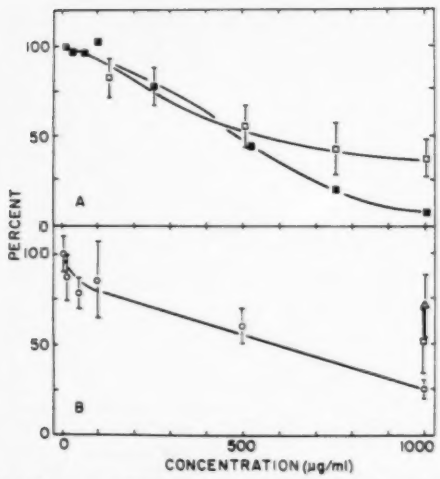

FIG. 7. Response of RAM (A) and CHO (B) cells to fluidized-bed combustion fine particulate (<3 μm) from flue gas. Data are mean values ± standard deviation (n = 3 or 6 for RAM assays; n = 5 for CHO assays). A: RAM cell ATP (fg/ml, percentage of control), □, 10% fetal calf serum, and ■, no fetal calf serum; and B: CHO colony survival, percentage of control, ○; washed particles, □, and filtrate, △.

Dose–response experiments were performed for each of the more toxic samples to further examine the sensitivity of the RAM and CHO assays. These data were used to determine by calculation an estimate of the sample concentration necessary to reduce cell viability, ATP, or cloning by 50% (EC_{50}). EC_{50} values are shown in Table 1. These data show that the concentration of particulate necessary

TABLE 1
EC_{50} VALUES AND CONFIDENCE INTERVALS FOR RAM PERCENTAGE VIABILITY, ATP, AND CHO COLONY SURVIVAL

Sample		EC_{50} (μg/ml concentration)[a]		
		Percentage viability	ATP[b]	Colony survival[c]
FBC: fine particulate	(10% FCS)	4566 (2272–20032)	596 (489–762)	474 (243–1421)
	(0 FCS)	392 (235–804)	329 (201–627)	—
FBC: fly ash	(10% FCS)	ND[d]	ND	—
	(0 FCS)	165 (117–231)	127 (79–190)	—
CCC: fly ash	(10% FCS)	3006 (1826–7295)	462 (395–547)	258 (223–297)
	(0 FCS)	195 (135–286)	200 (140–290)	—

[a] EC_{50} values were obtained by calculation from simple regression. The 95% confidence interval is represented by numbers in parentheses.
[b] ATP (fg/cell) % of control.
[c] Colony survival % of control.
[d] Not determinable (large extrapolations of the data that are not valid estimates of cytotoxicity).

to give a 50% response in cell viability was strongly influenced by serum protein. If serum was removed from the assay system, EC_{50} values for RAM viability and ATP were almost identical, and EC_{50} values for CHO colony survival were only slightly greater.

The cytotoxic effects of coal combustion and gasification particles in the RAM and CHO assays are summarized in Fig. 8; data for silica and TiO_2 are also presented. The data are plotted at either the dose for which parameters were depressed below the 50% level or below the 100% level at a 1000 μg/ml dose. Silica was the most toxic particle assayed; CCC fly ash was the most toxic of the coal related particles. Most of these particles exerted only small effects on cell viability, ATP, and cloning efficiency even at particle concentrations of 1000 μg/ml. However, these test systems did isolate some of the particles as more toxic. These data also illustrate the improved discrimination potential of the RAM system without serum.

DISCUSSION

In the preceding report, we showed that the RAM and CHO cell systems were useful and sensitive indicators of the toxicity of a variety of well-defined particulate substances and model fly ashes (Garrett *et al.*, 1981). The purpose of this

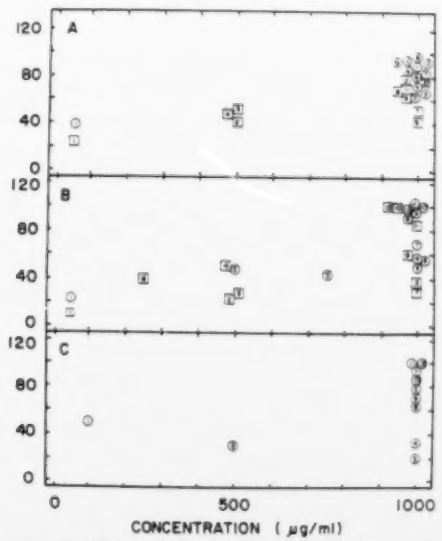

FIG. 8. Summary of cytotoxic effects of particulates in the RAM and the CHO clonal assay. The data points are plotted at the dose for which cell viability, ATP, or colony survival are depressed below the 50% level (or below the 100% level at the 1000 μg/ml dose). Control particles were (1) SiO_2, and (9) TiO_2. Sample (2) was a conventional coal combustion particle (<3 μm). Fluidized-bed combustion samples were: (3) stack gas fine particulate (<3 μm); (6) flue gas coarse particulate (>3 μm). (7) Coal feed; (4) fly ash; (5) spentbed; and (8) dolomite. Coal gasification samples were: (10) cyclone dust; (11) ash; (12) coal feed. A: RAM cell viability; B: RAM ATP (fg/cell, percentage of control); and C: CHO colony survival, percentage of control. ○, 10% fetal calf serum; □, no fetal calf serum.

paper has been to present data on the cytotoxicity of particles from coal-related processes which were assayed in an effort to evaluate and implement a test system for particulate emissions from industrial and energy-related sources. The particles evaluated consisted of a sample from a conventional coal-burning power plant, and samples from coal gasification and fluidized-bed combustion processes.

Cellular ATP was the most sensitive index of cytotoxicity of the coal-related samples in the standard RAM assay. Previous studies with silica, titanium dioxide, and lead oxide-coated fly ash (<2 μm) revealed that the sensitivity of the RAM assay could be enhanced if serum was removed from the culture media (Garrett *et al.*, 1981). The sensitivity of the assay was increased primarily by amplifying the effect on cell viability and ATP in treated cultures. Evaluation of the cytotoxicity of nine samples from coal-related processes also showed that the sensitivity of the assay was significantly improved by using serum-free media allowing better discrimination between individual samples.

A linear relationship was observed between percentage cell viability and ATP level in the particle-treated cultures. In the RAM assay without serum the slope of the line was 1.0, which provided strong evidence that the mechanism of toxicity was similar in reducing cell viability and ATP.

In other experiments dose–response curves were obtained for three of the more toxic particles using media with (10%) and without serum protein. These data, Table 1, showed that the concentration of particulate calculated to give a 50% response in cell viability (EC_{50}) was strongly influenced by the concentration of serum in the culture media. EC_{50} values for cellular ATP were less affected. EC_{50} values for cellular viability and ATP were found to be almost identical when the assay was conducted in serum-free media. The cause of the differential effects on cell viability and ATP in particle-treated cultures are unknown although it may be related to differences in cell membrane integrity in the presence of serum protein.

The assays without serum are most applicable to detection of particulate materials with low cytotoxicity. It may be argued that the serum-free system is unlike the cellular matrix of the lung, which is covered with a lipid- and protein-containing fluid. However, our results with a variety of particles indicate that both the serum-containing and serum-free assays select the more toxic particles although the sensitivities of the assays are quite different.

The CHO cloning assay was also shown to be a sensitive index of the toxicity of coal-related particles. The CHO system was essentially equivalent to the RAM assay without serum. Assay of RAM viability and ATP in serum-free media and CHO cell cloning were equally effective in selecting the more toxic particles. The agreement between cytotoxicity measured in the long-term (6-day) CHO assay and the short-term (20-hr) RAM assay without serum may possibly be explained by a loss of cell ATP which diminishes cell growth, yielding a correspondingly smaller number of cell colonies.

A summary of the data from assays of the coal-related particles revealed that many of the particles exerted little cytotoxicity at concentrations of 1000 μg/ml. Conventional coal combustion fly ash was the most toxic of the coal-related particles studied. The fluidized-bed combustion fly ash and fine particulate from stack gas also exhibited substantial cytotoxicity in the RAM and CHO assay

systems. These data show that more of the coal-related samples produced a toxic response at the lower particle concentrations when serum was deleted from the culture media.

It is noteworthy that the toxicity of particles from energy conversion processes is dependent on the fuel type and the plant operating and sample collection conditions. These data alone cannot be used to determine the hazard presented by particulate emissions from coal gasification or combustion processes.

The experiments reported here show that *in vitro* studies of toxicity at the cellular level with the rabbit alveolar macrophage and the Chinese hamster ovary cell can be used as a first stage of the biological evaluation of particulate materials obtained from coal-related processes. The cellular toxicity data can be coupled with other chemical and biological research to direct the utilization and improvement of pollutant control devices by detecting and ranking toxicity of effluents from these industrial processes.

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